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GERMINATION INHIBITORS IN AQUEOUS SEED EXTRACTS OF FOUR SOUTH AFRICAN PROTEACEAE. *

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Abstract

The seed of many species of Proteaceae germinate very poorly. Four species belonging to this family viz., *Protea compacta*, *P. barbigera*, *Leucospermum cordifolium* and *Leucadendron daphnoides* were studied in an attempt to determine the underlying causes of poor germination. Seeds were germinated under three temperature regimes with or without treatment with fungicide.

Water extracts of seed coats and embryos of all species, when applied to test seeds of lettuce (var. Grand Rapids) and water cress, either inhibited seed germination or the growth of seedling roots, or both. A comparison of the effect of a range of sucrose solutions of known osmotic pressure, and the seed extracts (osmotic pressure determined cryoscopically) on lettuce seed germination showed that inhibition of germination by seed extracts could not be accounted for on the basis of osmotic pressure alone. Chromatographic separation of the water extracts of seed of all species in *iso*-propanol: ammonia:water (10:1:1 v/v) indicated a band of inhibition corresponding to Rf values of 0,9 and 1,0. Abscisic acid and coumarin separated in the same solvent system produced inhibition in the bio-assays which could not be distinguished from that produced by the seed extracts.

Uittreksel

ONTKIEMINGS-INHIBEERDERS IN WATEREKSTRAKTE VAN SAAD VAN VIER SUID-AFRIKAANSE PROTEACEAE.

Die sade van baie soorte van die Proteaceae ontkiem swak. Gevolglik is 'n studie gemaak van die ontkieming van vier soorte in die familie nl., *Protea compacta*, *Protea barbigera*, *Leucospermum cordifolium* en *Leucadendron daphnoides*, om te probeer vasstel wat die grondliggende oorsaak vir die swak ontkieming is. Die ontkieming van die saad is ondersoek by verskillende temperatuur-toestande in die aan- of afwesigheid van 'n swamdoder.

Die aanwending van waterekstrakte van saadhuide en embrios van al vier soorte, het óf die ontkieming, óf die wortelgroei van saailinge, óf beide, van slaai- (var. Grand Rapids) sowel as bronkhors-saad geïnhibeer. 'n Studie van die effek van 'n reeks sukrose-konsentrasies met bekende osmotiese druk en saadekstrakte (osmotiese druk krioskopies bepaal) op slaaisaadontkieming, het aan die lig gebring dat die inhibisie van ontkieming nie geheel-en-al aan die osmotiese druk van die saadekstrakte toegeskryf kan word nie. Chromatografiese skeidings van waterekstrakte van al vier spesies in *iso*-propanol: ammonia:water (10:1:1 v/v) het inhibisie getoon by Rf-waardes van 0,9 en 1,0. Absissiensuur en koumarien, geskei in dieselfde oplosmiddel, het inhibisie getoon in die biotoetse, wat nie van dié van die saadekstrakte onderskei kon word nie.

Introduction

One of the major obstacles to the large-scale cultivation of members of the South African Proteaceae is the fact that many are difficult to propagate.

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Although vegetative propagation by means of cuttings has proved successful and has certain advantages (Salinger, 1964; Rousseau, 1965, 1967; Topper, 1966) most plants are still raised from seed because the latter method requires relatively unspecialized facilities. The seed of many species, however, germinates very poorly, a fact long known to growers. Unfortunately, much of the literature on the propagation of members of the Proteaceae by seed is of a popular nature and is not very specific as to the treatments that have been used in attempts to improve germination (Thorns, 1943; Werner, 1951; Vogts, 1960).

Some preliminary laboratory work on seed germination in a number of species has been done (Atkinson, 1961; Horn, 1962; van Staden, 1966), but no clear-cut answers to the problem of poor germination have been obtained. Atkinson (1961) conducted investigations into seed germination in *Leucospermum catherinae*, but as very small samples of seeds were used, interpretation of her results is often very difficult. Horn (1962) maintained that poor germination in the Proteaceae was the result of ineffective seed screening methods. However, in his investigations and those of van Staden (1966) more effective seed screening methods were used and these still gave samples with a low percentage germination. Vogts (1960) suggested that the poor germination obtained in samples of proteaceous seeds was due to the presence of germination inhibitors within the seeds. She maintained that these inhibitors could be leached out, if the seeds were watered regularly. Horn (1962) did not regard germination inhibitors as being of importance in preventing germination of seed of this family.

Against the background of these conflicting experimental results and opinions, a series of investigations was initiated with the aim of determining the underlying causes of poor seed germination.

MATERIALS AND METHODS

Plant material and germination techniques. An investigation was made of the germination of seed of *Protea compacta* R.Br., *Protea barbigera* Meisn., *Leucospermum cordifolium* (Salisb. ex Knight) Fourcade {= L. *nutans* R.Br.} and *Leucadendron daphnoides* Meisn. These particular species were chosen for study as they all have relatively large seeds which are more effectively sorted than the smaller sized seeds of many other species. Seed was carefully sorted and only apparently sound, undamaged seed was used in experiments. Seed used was purchased from the Department of Forestry, Pretoria and from M. J. Middelman, Newlands, Cape, South Africa.

At the outset of the investigation it was necessary to obtain some indication of the effect of temperature on the germination of seeds. Two hundred seeds of each species was germinated in growth cabinets under the three temperature regimes available to the authors. These were (i) alternating temperatures of 15°C for 16 hours followed by 20°C for eight hours; (ii) alternating temperatures of 20°C for 16 hours, followed by 30°C for eight hours; and (iii) a constant temperature of 26°C. In (i) and (ii) light (ca. 1 lm/m^2) was supplied by cool white fluorescent tubes to coincide with the higher temperature period and in (iii) no light was supplied.

Seed was germinated in petri dishes on acid-washed sand. Distilled water was added to the sand until it was just moist and this level of moisture was maintained by the addition of further water when necessary. In order to test the effect of a fungicide treatment on germination, half the seeds of each species were soaked in 5% "Kaptan" solution for 30 minutes before being placed in the petri dishes. The criterion for germination was taken as the emergence of a healthy radicle approximately two mm in length. Germination counts were made daily for 63 days and seeds were removed from the petri dishes on germinating.

Leaching of Seeds. In order to determine whether a water-soluble inhibitor was present, 15 g of seed of each species was separated into two components viz., seed coats and embryos (which included cotyledons) and each component (unground) was shaken in 100 ml distilled water for 12 hours at room temperature. The leachate was filtered and the filtrate concentrated to 12 ml in a rotary flash evaporator at 40°C under reduced pressure. The inhibitor activity of the extracts was then tested in the lettuce seed and water cress seed bioassays.

Chromatography. Where extracts were separated this was done at room temperature using descending paper chromatography. The concentrated extracts were strip loaded on Whatmans No. 1 chromatography paper. The chromatograms were equilibrated for four hours prior to development. After the solvent front had travelled about 40 cm, the chromatograms were dried and cut into ten equal strips. The biological activity of each strip was determined in the lettuce seed bioassay. The extracts were separated in *iso*-propanol:ammonia: water (10:1:1 v/v).

Bioassays. At first it appeared that the ideal material for use in bioassays for the presence of germination promotors or inhibitors would be the excised embryos of the seed of each species. However, several attempts to grow these embryos on either filter paper or sand were not successful. Atkinson (1961) also failed in her attempts to germinate excised embryos of seed of *Leucospermum catherinae*. It was therefore decided to use the lettuce and cress seed bioassays.

(i) *Lettuce seed germination*. The influence of seed coat and embryo extracts on the germination of lettuce seed (Grand Rapids obtained from J. E. Ohlsen's Enke, Denmark) was investigated using the techniques of Sankhla and Sankhla (1968) and Wurzburger and Leshem (1969). Three millilitres of seed extract or distilled water (in the case of controls) were added to 50 lettuce seeds on two layers of Whatman No. 1 filter paper in a petri dish. Each treatment was replicated four times. After the seed extracts or distilled water were added to the lettuce seeds, the latter were kept in the dark for two hours. The lettuce seeds were then exposed to cool white fluorescent light with an intensity of ca. $3{,}0 \times 10^2$ 1 m/m^2 for 30 mins. After illumination, the seeds were germinated in the dark in a growth cabinet maintained at 26°C. After 48 hours the percentage germination was recorded and the length of the root of each seedling was measured.

(ii) *Cress seed germination.* The cress seed germination test was also used to bioassay the seed extracts. The procedure followed was adapted from that of Valio and Schwabe (1970). Three millilitres of seed extract or distilled water (in the case of controls) were added to 25 cress seeds on two layers of Whatman No. 1 filter paper in a petri dish. Each treatment was replicated four times. Seeds were germinated in the dark in a growth cabinet at 26°C. Seeds were regarded as having germinated once the root tip had penetrated through the gelatinous layer surrounding the seed coat. After 48 hours the percentage germination was recorded and the length of the root of each seedling was measured.

RESULTS AND DISCUSSION

Germination results after 63 days are shown in Table 1. In *Protea compacta*, *Leucospermum cordifolium* and *Leucadendron daphnoides* the germination percentage was significantly higher at 15°—20°C than at 20°—30°C. The same trend was shown by *Protea barbigera*, although differences were not statistically significant. Results for *P. cynaroides*, which were included for purposes of comparison, show indications of the opposite trend. However, differences were not statistically significant. It is interesting to note that the only seeds to ger-

TABLE 1

The effect of temperature on germination of seed of *Protea compacta, P. barbigera, P. cynaroides, Leucospermum cordifolium* and *Leucadendron daphnoides*.

Species	Temperature		Difference between temperatures
	15—20°	20—30°	
	Transformed means*	Transformed means*	
Protea compacta	28,45	18,04	10,41†
P. barbigera	32,07	26,22	5,85 N.S.
P. cynaroides	27,32	30,97	3,65 N.S.
Leucospermum cordifolium	25,94	4,93	21,0‡
Leucadendron daphnoides	34,32	15,42	18,90‡

* Angular transformation of percentages to degrees.
N.S. = Non significant.
† = Significant at 5% level.
‡ = Significant at 1% level.

minate at a constant temperature of 26°C were those of *P. cynaroides* (3 %). The fungicide treatment brought about a reduction in germination in all species, but the effect was not statistically significant.

A relatively large proportion of the seeds failed to germinate. There are three possible reasons for this. Firstly, temperatures may not have been optimum for germination. Secondly, a large proportion of the seeds may have been infertile, possibly due to ineffective seed sorting. Thirdly, the seed may have been dormant due to the presence of an endogenous inhibitor. The first possibility cannot be ruled out completely because of the lack of data on optimum germination temperatures for the seed of South African Proteaceae. The second possibility appears to be unlikely as the seeds which failed to germinate by the end of the experiment were cut open and depending on species only 5—11 % of the seeds did not contain fully formed embryos. The most likely explanation for the poor germination appears to be that the seeds are dormant due to the presence of endogenous inhibitors or a lack of promotors, or both.

The presence of inhibitors was indicated in a study of the effect of the application of water extracts of seed coats and seed embryos on the germination and seedling root growth of Grand Rapids lettuce seed and cress seed (See Tables 2 and 3.)

Embryo extracts of *Protea compacta*, *P. barbigera* and *Leucospermum cordifolium* gave almost complete inhibition of germination when applied to test seeds of lettuce and cress. The application of the embryo extract of *Leucadendron daphnoides* gave germination results significantly (1 % level) below those of the distilled water controls.

TABLE 2

The effect of water extracts of seed of *Protea compacta* and *P. barbigera* on seed germination and seedling root growth of water cress and Grand Rapids lettuce.

Seed extract	Water cress		Grand Rapids lettuce	
	Seed germination	Seedling root growth	Seed germination	Seedling root growth
	Transformed means*	mean length (mm)	Transformed means*	mean length (mm)
Control (distilled water) .	59,88	13,20	80,20	11,22
P. compacta coat . . .	55,60	8,12	70,80	2,45
†*P. compacta* embryo . .	complete inhibition	complete inhibition	complete inhibition	complete inhibition
P. barbigera coat . . .	54,40	5,20	56,90	1,52
†*P. barbigera* embryo . .	complete inhibition	complete inhibition	complete inhibition	complete inhibition
Least significant P(0,05) .	9,69	0,73	6,57	0,84
differences P(0,01) .	14,68	1,11	9,97	1,27

* Angular transformation of percentages to degrees.

† Where germination was zero, treatments could not be included in a statistical analysis.

TABLE 3

The effect of water extracts of seed of *Leucospermum cordifolium* and *Leucadendron daphnoides* on seed germination and seedling root growth of water cress and Grand Rapids lettuce.

Seed extract	Water cress		Grand Rapids lettuce	
	Seed germination	Seedling root growth	Seed germination	Seedling root growth
	Transformed means*	mean length (mm)	Transformed means*	mean length (mm)
Control (distilled water) .	60,40	15,83	73,18	21,20
Leucospermum cordifolium coat	2,88	0,50	18,53	2,25
†*L. cordifolium* embryo .	2,88	0,25	Complete inhibition	Complete inhibition
Leucadendron daphnoides coat	33,93	2,43	58,55	3,85
L. daphnoides embryo . .	37,33	2,38	57,35	6,28
Least significant differences P(0,05) .	10,53	1,74	8,62	1,17
Least significant differences P(0,01) .	14,77	2,43	12,39	

* Angular transformation of percentages to degrees.

† Where germination was zero, treatments could not be included in a statistical analysis.

Water extracts of seed coats, in general, did not give such marked inhibition, but nevertheless germination results were still significantly below those of the distilled water controls. The seed coat extract of *Leucospermum cordifolium*, however, was the exception and gave particularly marked inhibition of germination in both bioassays. It is also apparent that the seed coat extract of *Protea compacta* brought about less inhibition of germination than the seed coat extracts of *P. barbigera* and *Leucadendron daphnoides*.

The fact that the seed coat and embryo extracts of all species had, to some extent, an inhibitory effect on lettuce and cress seed germination and seedling root growth suggested that in addition to the possibility of the presence of a chemical inhibitor, the osmotic pressure of the extracts might be exerting an inhibitory effect. In order to obtain some indication of the effect of osmotic pressure on the germination of lettuce seeds, samples were germinated on filter paper moistened with sucrose solutions ranging in molarity from 0,025 M to 0,4 M (corresponding to osmotic pressures ranging from 0,66 bars to 11,2 bars) (See Figure 1). This figure shows a curve very similar to that obtained by Lerner, Mayer, and Evenari (1959). These workers germinated lettuce seed on a range of NaCl solutions giving osmotic pressure values from 0,5 to 8,1 bars. Whereas they obtained complete inhibition of germination at O.P = 6,1 bars, in the present study complete inhibition of germination occurred at O.P. = 8,1 bars.

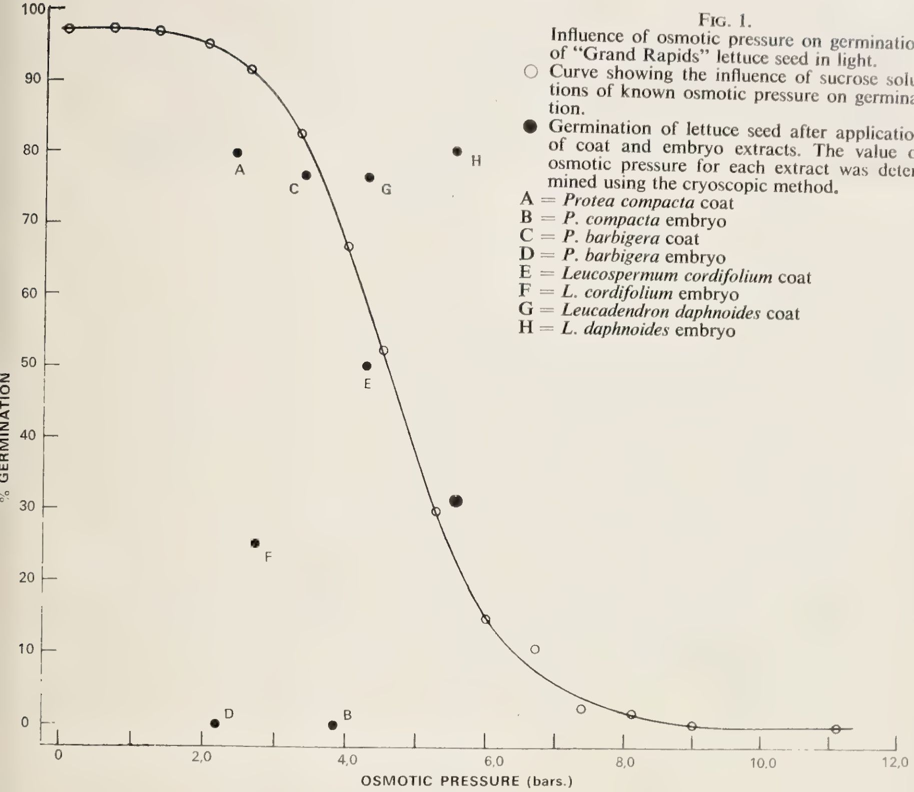
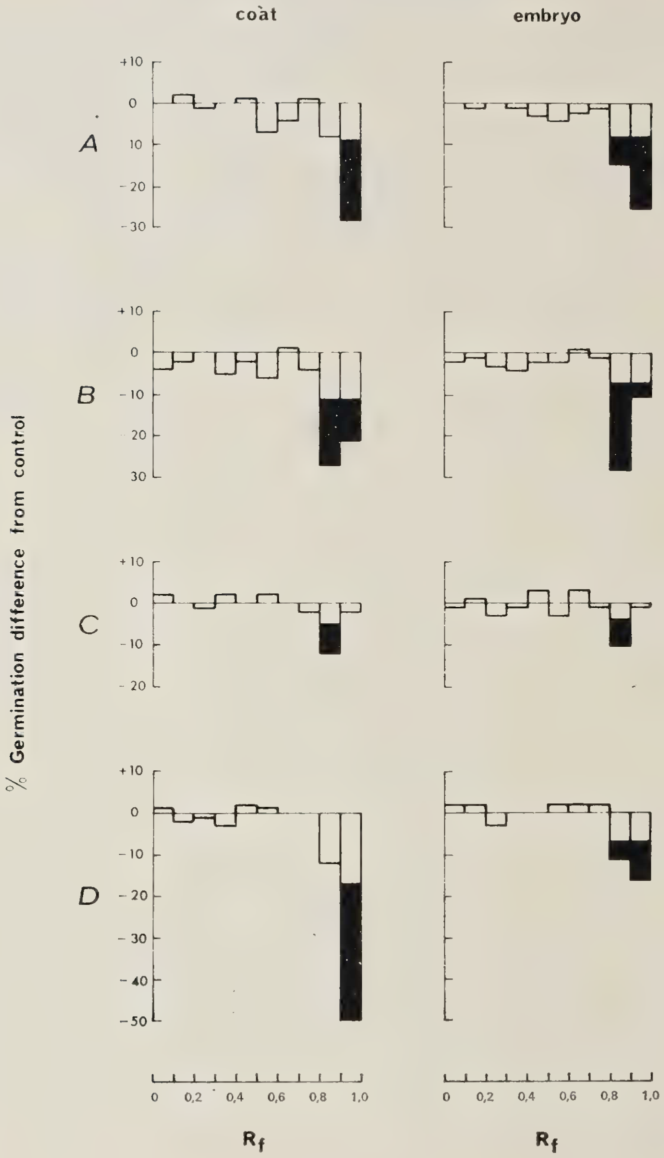


FIG. 1.
Influence of osmotic pressure on germination of "Grand Rapids" lettuce seed in light.
○ Curve showing the influence of sucrose solutions of known osmotic pressure on germination.
● Germination of lettuce seed after application of coat and embryo extracts. The value of osmotic pressure for each extract was determined using the cryoscopic method.
A = *Protea compacta* coat
B = *P. compacta* embryo
C = *P. barbigera* coat
D = *P. barbigera* embryo
E = *Leucospermum cordifolium* coat
F = *L. cordifolium* embryo
G = *Leucadendron daphnoides* coat
H = *L. daphnoides* embryo

The osmotic pressure of each seed extract was determined using the cryoscopic method. These seed extracts were then applied to lettuce seed which was treated as in the bioassay. Seed extract osmotic pressure values and the corresponding lettuce seed germination results were then plotted on Figure 1.

Six of the eight seed extracts gave germination results below those obtained with sucrose solutions of equivalent osmotic pressure. The remaining two, viz the seed coat and seed embryo extracts of *Leucadendron daphnoides*, gave germination results slightly above those of the sucrose solution of equivalent osmotic pressure. Table 3, shows that these latter two extracts, although not influencing germination, to the same extent as the other seed extracts did reduce seedling root growth significantly (1 %).


coat
embryo
% Germination difference from control
A
B
C
D
+10
0
10
- 20
- 30
- 10
20
30
- 40
- 50
0
0,2
0,4
0,6
0,8
1,0
Rf

All the extracts have values of osmotic pressure which fall within the range of 0,56—6,1 bars, which Lerner and Evenari (1961) designate "the region of combined osmotic pressure and chemical inhibition".

This tends to indicate that the results in Tables 2—3 and Figure 1 cannot be explained on the basis of osmotic pressure alone and that there appears to be one or more chemical substance present in the seed extracts, which either inhibits germination or seedling root growth or both.

Further evidence of the presence of an inhibitor was shown in the bioassay of the chromatographed seed extracts. Figure 2 shows the lettuce seed bioassay results after the separation of the extracts in *iso*-propanol:ammonia:water. The seed coat and seed embryo extracts of all species showed a band of inhibition corresponding to Rf values 0,9—1,0. These results were compared with the inhibitory effects of two widely occurring germination inhibotors viz. coumarin and abscisic acid (ABA) which were separated in the same solvent system (Fig. 3). The coumarin standard alone gave a band of inhibition corresponding to Rf values 0,9—1,0 and the ABA standard alone gave inhibition at Rf 0,8—0,9. A mixture of the two standards gave inhibition at Rf 0,8—0,9. Thus in this solvent system the inhibitory effects of the two standards could not be distinguished and the band of inhibition shown by the seed extracts corresponded with both ABA and coumarin.

Germination inhibitors have thus been shown to be present in the seed extracts and the possibility exists that they may play a role in the regulation of seed germination under natural conditions. There are also some indications that the inhibitors present in the seed extracts have properties similar to either ABA or coumarin or both. Further work remains to be done to characterize the inhibitor(s) more clearly.

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FIG. 2.

A comparison of the effects of water extracts of seed coats and embryos on germination of "Grand Rapids" lettuce seed in light.

A = *Protea compacta*
B = *P. barbigera*
C = *Leucospermum cordifolium*
D = *Leucadendron daphnoides*

The solvent was *iso*-propanol:ammonia:water. Shaded areas represent differences significant at 1% level.

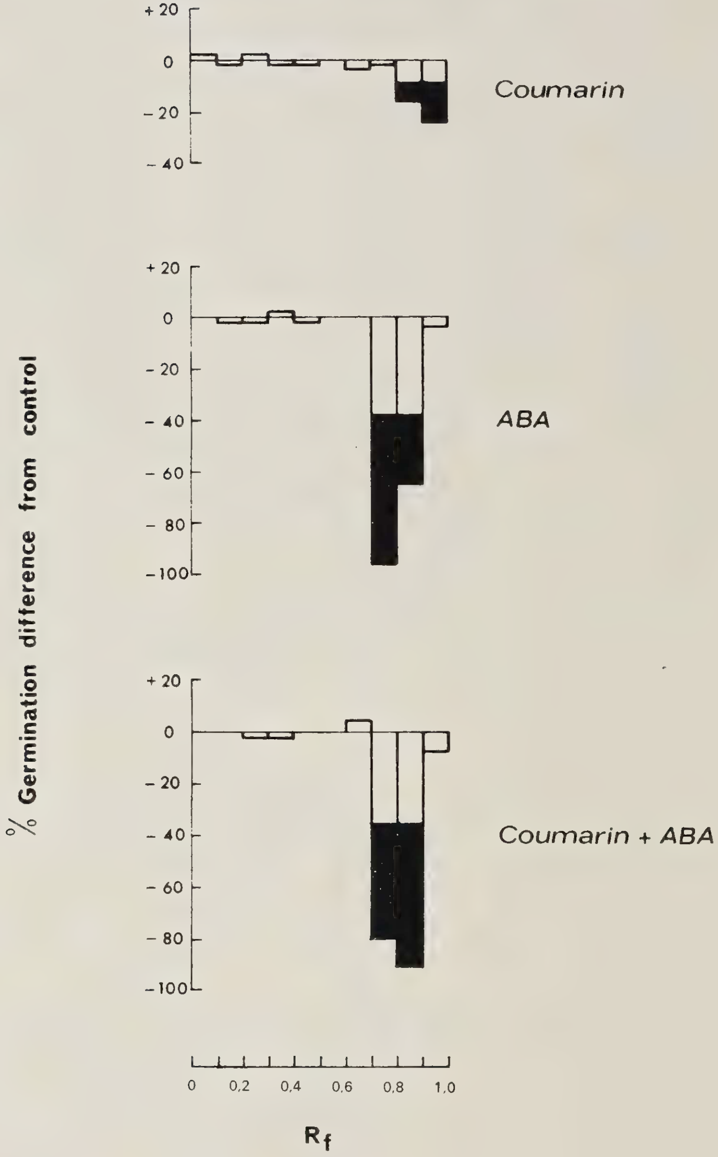
+ 20
0
− 20
− 40
Coumarin
+ 20
0
− 20
− 40
− 60
− 80
− 100
ABA
+ 20
0
− 20
− 40
− 60
− 80
− 100
Coumarin + ABA
% Germination difference from control
0
0,2
0,4
0,6
0,8
1,0
R_f

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FIG. 3.

A comparison of the effects of ABA and coumarin on the germination of "Grand Rapids" lettuce seed in light. The solvent was *iso*-propanol:ammonia:water. The shaded areas represent differences significant at the 1% level.